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RESEARCH ARTICLE

Number Density of Mast Cells in the Primo Nodes of Rats

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Abstract
Mast cells (MCs) play a major role in allergic reactions. Surprisingly, the acupuncture points have a higher density of MCs compared with nonacupoints in the skin, which is consistent with the augmentation of the immune function by acupuncture treatment. We hypothesized that the primo vascular system (PVS), which was proposed as the anatomical structure of the acupuncture points and meridians, should have a high density of MCs. In order to test that hypothesis, we investigated the primo nodes isolated from the surfaces of internal organs, such as the liver, the small and the large intestines, and the bladder. The harvested primo nodes were stained with toluidine blue, and the MCs were easily recognized by their red–purple stains and their characteristic granules. The results showed a high density of MCs in the primo nodes and confirmed the hypothesis. The MCs were uniformly distributed in the nodes. The relative concentration of the MCs with respect to other cells was ~15%. We divided the sizes of the primo nodes into three classes: large, medium, and small. The number density and the

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relative concentration of MCs did not show a size-dependence. The current work suggests that the PVS may participate in the immune response to allergic inflammation, which closely involves MCs.

1. Introduction

Allergic diseases are increasing in prevalence and among the commonest causes of chronic ill-health worldwide. In order to reduce the burden of health care costs alternative medicines such as acupuncture are increasingly tried besides conventional Western medicine. Augmentation of the immune function is one of the widely-acknowledged effects of acupuncture treatment [1]. The active carriers of the defense mechanism are immune cells such as mast cells (MCs), neutrophils, eosinophils, basophils, macrophages, and lymphocytes. Among these, MCs are closely related to acupuncture treatments: There are more MCs at acupoints and acupuncture meridians than at nearby nonacupoints [2]; electro-acupuncture induces more MCs at other acupoints [3,4]. In addition, stimulation of the acupoints results in a significant increase in the degranulation of the mast cells [5].

Bong-Han Kim (BH Kim) [6], the first discoverer of the new circulatory system, the so-called primo vascular system (PVS) claimed that the anatomical structure corresponding to the classical acupuncture meridians was a subsystem of the PVS distributed in the skin of animals and humans. However, there has been no confirmation of his claim, and only one paper has presented a negative result [7]. Even though that is not direct proof of BH Kim's claim, the high concentration of MCs in the PVS could be thought of as supportive data for his claim because MCs are more highly concentrated at acupoints than at nonacupoints.

The presence of immune cells, such as MCs, macrophages, neutrophils, and others, were observed in the PVS on the surfaces of internal organs of rats [8] and in the primo nodes (PNs) obtained above the epicardia of rat hearts [9].The MCs amounted to up to 20% of residential cells in the PNs harvested from the surfaces of internal organs and from the insides of lymphatic vessels of rats [10]. However, another count of the MCs reported only ~4% in the PNs on the surfaces of internal organs of rats [11].The reason the relative number density shows such relatively large differences is still not clearly understood. Therefore, investigations of the density of MCs by independent teams are worth doing.

In the current work, we report on the number density and the relative concentration of the MCs in the PNs on the surfaces of internal organs of rats. With toluidine-blue staining, the MCs were easily distinguishable from other types of cells, and the areas of the PNs could be determined by counting the number of pixels in charge coupled device (CCD) images (Microscope Digital Camera, (DP70, Olympus, Tokyo Japan)). We classified the PNs into three groups, large (L), medium (M), and small (S), according to the areal-sizes of the middle sections of the PNs. We hypothesized that either the absolute number or the relative density of the MCs depended upon the sizes of the PNs.

MCs are bone marrow-derived and particularly depend on the stem-cell factor for their survival. They are found to reside in tissues near blood vessels and nerves and in proximity to surfaces that interface with the external environment [12]. MCs, vascular vessels, and nervous fibers form a composite structure. MCs were found to be migrated and recruited in the acupoints and meridians by acupuncture stimuli [13].The biological functions of MCs appear to include roles in host defense mechanisms against pathogens, in tissue repair, and in angiogenesis. MCs may be activated by various stimuli, and, after activation, MCs may extrude granules that contain histamine and induce allergic inflammation [12]. Some of the MCs were observed to degranulate in the PNs [8] and in the pericardial space [9]. Substantial numbers of granules from MCs were found in primo subvessels [10], suggesting that primo vessels are conduits for the granules.

MCs have emerged as a connection between Western physiology and Eastern acupuncture via the new circulatory system, the PVS. In terms of Western physiology, the positive effects of acupuncture on the immune function can be explained through a combination of MCs and the PVS.

2. Materials and methods

2.1. Animal preparation and harvesting of the Organ Surface-Primo Node (OS-PNs)

Ten male Sprague-Dawley (SD) rats (7—9 weeks of age) were obtained from DooYeol Biotech (Seoul, Korea). The animals were housed in constant temperature and humidity conditions (23°C, relative humidity 60%) with a 12-hour/12-hour light/dark cycles and were provided water and commercial rat chow *ad libitum*. The procedures involving the animals and their care were in full compliance with current international laws and policies and were approved by the Institutional Ethics Committee of the Advanced Institute of Convergence Technology, Seoul National University (Approval Number: WJIACUC20140807-03-07). The rats were anesthetized by using an intramuscular injection of a regimen consisting of 1.5 g/kg of urethane and 20 mg/kg of xylazine. The rats were sacrificed by using an intracardiac injection of 1 mL of urethane after the experiments.

An incision of the subcutaneous layer of the abdominal skin along the midline, but slightly off the linea alba, was performed with surgical scissors. We avoided cutting the linea alba in order to maintain the abdominal wall fat band located at the midline of the ventral peritoneal wall because some PNs are often found in the abdominal wall fat band. All procedures of observations and operations were performed under a stereomicroscope (SZX12, Olympus, Tokyo, Japan). We searched for the Organ Surface-PVS (OS-

PVS) in the abdominal cavity under a stereomicroscope (STZ10, Olympus, Tokyo, Japan). During the procedure, avoiding blood flow into the abdominal cavity and keeping the surfaces of internal organs humid by dripping phosphate buffer solution (PBS) onto them frequently were important.

2.2. Toluidine blue staining

2.2.1. Paraffin section

The isolated organ surface PNs (OS-PNs) were fixed with neutral buffered formalin (NBF) at 23°C for 24 ± 2 hours. The specimens were embedded in paraffin and cut to 5-μm-thick sections by using a microtome (Reichert-Jung 820 Histocut Microtome, Leica Wetzlar, Germany). The sections at the middle and off the middle of the OS-PNs were studied.

2.2.2. Toluidine blue staining

The toluidine-blue stock solution was made by melting 0.1 g of toluidine-blue powder (Toluidine blue O, 198161-5G, Sigma-Aldrich, St. Louis, MO, USA) and 10 mL of 70% alcohol. The working solution at pH 2.3 was made by mixing the stock solution with sodium chloride (1%, pH 2.3). The specimens were stained with toluidine blue for 60 ± 20 seconds. They were dehydrated by dipping them quickly 10—15 times first, in 95% and next in 100% ethanol. They were then dipped in xylene for 3 minutes, which was repeated. They were mounted on a glass slide.

2.2.3. Counting of mast cells

The stained specimens were observed under a phase contrast microscope (BX51, Olympus) to count the MCs, which were easily recognizable because of their stained red—purple (metachromatic staining) color and the blue-colored background. The granules from the MCs, which were often scattered around the MCs, were a prominent signature of the MCs. The areas of the OS-PNs were measured by using the Image J and the Tsview-programs and counting the numbers of pixels. The size of a pixel was $0.64 \times 0.64\ \mu m^2$.

3. Results

The experimental data are summarized in Table 1. In brief, the sections of 10 large-size PNs with areas of $272.6 \times 10^3 \pm 105.3\ \mu m^2$ were examined after toluidine-blue staining, and the number density of MCs was 1.9 ± 0.8 per $10^3\ \mu m^2$. The area of eight middle-size PNs was $95.3 \pm 17.8\ \mu m^2$, and the MC density was 2.2 ± 1.2 per $10^3\ \mu m^2$. The area of five small-size PNs was $25.6 \pm 10.2\ \mu m^2$, and the MC density was 2.3 ± 1.3 per $10^3\ \mu m^2$.

Fig. 1A shows the locations where the 23 OS-PNs were harvested. They were scattered throughout the abdominal cavity. Fig. 1B shows a large-size PN and a vessel around the large intestine. It was harvested and put on a slide glass, as shown in Fig. 1C. The PN had a long cucumber shape with

Table 1 Number density of mast cells in a primo node.

Size class of PN (1,000 μm^2)	Rat	No. of MCs	Area of PN (1000 μm^2)	No. density (n/1000 μm^2)
L (A > 150)	1	500	216	2.3
	2	273	282	1.0
	3	510	259	2.0
	4	238	315	0.8
	5	579	259	2.2
	6	265	181	1.5
	7	427	370	1.2
	8	856	506	1.7
	9	588	165	3.6
	10	375	173	2.2
Av ± SD		461.1 ± 189.2	272.6 ± 105.0	1.9 ± 0.8
M (50 < A < 150)	1	93	88	1.1
	2	54	104	0.5
	3	210	63	3.3
	4	360	94	3.8
	5	261	92	2.8
	6	154	90	1.7
	7	132	106	1.2
	8	350	125	2.8
Av ± SD		201.8 ± 114.3	95.3 ± 17.8	2.2 ± 1.2
S (A < 50)	1	74	17	4.3
	2	46	32	1.5
	3	77	31	2.5
	4	16	13	1.2
	5	60	37	1.6
Av ± SD		54.6 ± 24.9	26 ± 10.4	2.2 ± 1.3

Av = average, MC = mast cell; PN = primo node; SD = standard deviation.

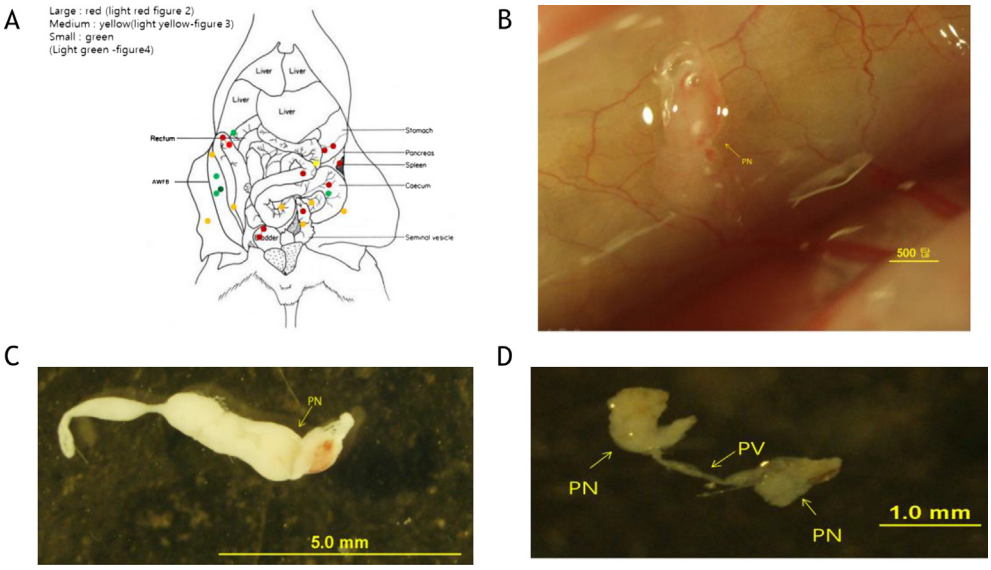

Figure 1 (A) Locations where primo nodes were taken for the current study. The OS-PVS formed a network on the surfaces of abdominal organs. PNs with cucumber shapes of various sizes were floating in the peritoneal fluid in the abdominal cavity: red, large-size PNs, orange, medium-size PNs, and green, small-size PNs. (B) Large-size OS-PN (arrow) around the small intestine of a rat. This is an *in vivo in situ* stereomicroscopic image. (C) The OS-PN in (B) was harvested and put on a petri dish. (D) Another specimen of a medium-size OS-PN which was harvested from around the small intestine. Two PNs (arrows) were connected by a primo vessel. OS-PN = organ surface primo node; OS-PVS = organ surface primo vascular system; PN = primo node; PV = primo vessel.

dimensions of 0.8 mm × 4.3 mm. Fig. 1D shows another specimen of two middle-size PNs harvested around the small intestine. They were connected by a primo vessel whose length was 1.1 mm.

The MCs were clearly distinguished from other cells due to the red—purple (metachromatic staining) color of toluidine-blue staining. Representative examples of the stained results for large-, medium-, and small-size PNs are presented in Figs. 2—4, respectively.

Fig. 2A shows an example of a large-size OS-PN harvested from the surface of the small intestine. Figs. 2A and 2B show a section at the middle of the PN after it had been stained with toluidine blue and a magnified view of the rectangular area in Fig. 2A, respectively. The area of the middle section was 173 × 10^3 μm^2, the MC count was 375 and the MC number density was 2.2 per 10^3 μm^2.

Figs. 3A and 3B, which are for a midsize PN around a small intestine, show the middle section of the PN with an area of 106 × 10^3 μm^2 after it had been stained. The MC count was 132, and the MC number density was 1.2 per 10^3 μm^2. Similarly, Figs. 4A and 4B depict a small-size PN on the ventral abdominal wall and its midsection image after toluidine-blue staining, respectively. Its area, MC count, and MC number density were 13 × 10^3 μm^2, 16, and 1.2 per 10^3 μm^2, respectively.

As shown in Figs. 2—4, the distributions of MCs were somewhat uniform around each section. They were not concentrated at either the boundaries or the centers of the PNs. We examined sections off the middle for some PNs and found the number density of MCs to be 1.4 per 10^3 μm^2, which was not significantly different from the values for the middle sections. This is consistent with the uniform distribution of mast cells within the middle sections.

We also counted the total numbers of cells in the middle sections of nine PNs in order study the relative density of MCs compared with the total number of cells. The ratio of

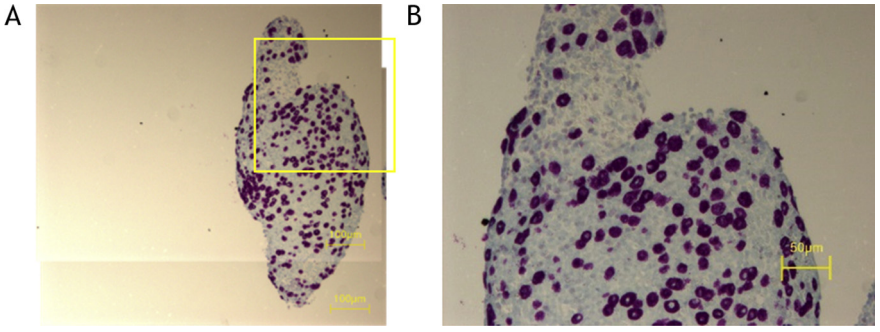

Figure 2 Large-size OS-PN around the small intestine of a rat. (A) Cross section in the middle of the PN stained with toluidine blue after paraffin embedding (100×). The scale baris 100 μm. (B) Magnified view of the rectangular area (200×). The scale bar is 50 μm. The mast cells could clearly be distinguished from other cells by using toluidine blue staining. OS-PN = organ surface primo node; PN = primo node.

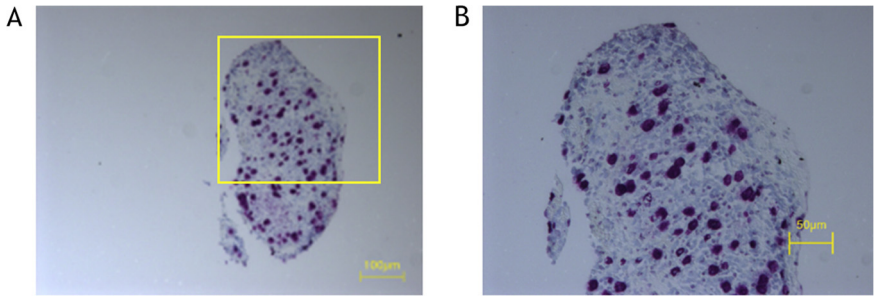

Figure 3 Medium-size OS-PN around the small intestine of a rat. (A) Cross section in the middle of the PN stained with toluidine blue after paraffin embedding (100×). The scale bar is 100 μm. (B) Magnified view (200×). The scale bar is 50 μm. OS-PN = organ surface primo node; PN = primo node.

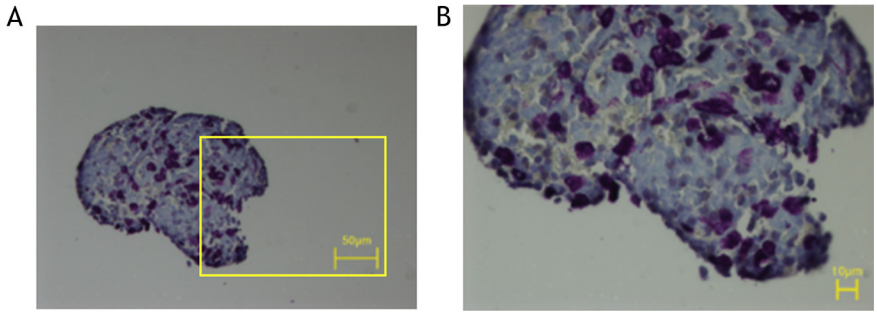

Figure 4 Small-size OS-PN on the abdominal wall of a rat. (A) Middle section after toluidine staining (200×). The scale bar is 50 μm. (B) Magnified view (400×). The scale bar is 10 μm. OS-PN = organ surface primo node.

the number of MCs to the total number of cells was 15.0 ± 5.2%.

4. Discussion

The absolute number density of MCs we observed was ~2,200/mm^2 in the OS-PN of a rat. This was 6.4 times higher than that of the acupuncture point (ST-36) in the skin of a rat. The reported density in the skin is 341.9 ± 104.3/mm^2, and that in a neighboring nonacupoint is 186.8 ± 81.5/mm^2 [4], which are comparable to those in another previous report, 588 ± 23/mm^2 and 78 ± 28/mm^2, respectively [2]. In the case of humans, the number densities of MCs were 31/mm^{2}and 210/mm^2 for 10-μm-thick sections in the skin and in the mucosa of the small intestine, respectively [14]. The ratio between the number density of MCs in the intestinal mucosa and that in the skin was 6.8, which is close to that of a rat. Interestingly, the ratios are in good agreement whereas the number densities of MCs vary widely depending upon the location and the species.

The relative number density among the residential cells in PNs was ~15%, which was in good agreement with the 20% reported previously [10], but > 4% [11]. The difference may be due to the techniques used or to physiological variations on the rats. Further research is needed to clearly understand the variation in relative density of MCs in PNs.

We classified the sizes of the OS-PNs according to the areas of their midsections: The areas of the large (L), middle, and small PNs were defined as areas > 150 × 10^3 μm^2, areas between 150 × 10^3 μm^2 and 50 × 10^3 μm^2, and areas < 50 × 10^3 μm^2, respectively. This definition of PN sizes was a subjective one for convenience of classification and was based on our experience with the sizes of the OS-PNs. The sample quantities shown in Table 1, 10 large-, eight middle-, and five small-size PNs, does not represent the relative distribution of the sizes of the OS-PNs; they also include the skills of the experimenters. For example, the yields of the histological analysis of the small-size PNs were much lower than those of the large-size PNs.

We hypothesized that either the absolute number or the relative density of MCs might depend upon the sizes of the PNs. However, the results were contrary to our anticipation. No significant PN-size dependence was found. In addition, the distributions of MCs within the OS-PNs were rather uniform around the midsections of the specimens. This was consistent with the observation that the number density in the off-mid sections was similar to that of the midsections. This size-independence and uniformity of the MC distribution within an OS-PN may provide some information for answering questions regarding the way in which MCs move to PNs or the way in which they are generated in PNs. This would be worthwhile investigating in the future.

With respect to acupuncture, an early report indicated that the MCs were more concentrated under the meridian lines that they were in control areas in humans and rats [2]. Subsequently, the number densities of MCs at acupoints were shown to be higher than those in the neighborhoods of the acupoints, and electro-acupuncture was shown to increase the MCs at other acupoints [3,4]. In addition, acupuncture raised the degree of degranulation of the mast cells in the acupoint area [5]. Furthermore, moxibustion at the acupoint ST25 in rats with colitis activated mast cell degranulation [15].

High concentrations of MCs were observed in the PNs harvested from the surfaces of the internal organs and from the lymph vessels of rats [8—11]. Also, degranulation of the MCs was observed in OS-PNs [8], and the flow of the granules in the channels of a primo vessel was inferred by using confocal 3D images [10]. These results are consistent with BH Kim's claim that the PVS is an anatomical structure of acupuncture points and meridians.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

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